Neuron 49, 389–401, February 16, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.neuron.2006.01.018

The Zinc Finger Transcription Factor Sp8 Regulates the Generation and Diversity of Olfactory Bulb Interneurons

Ronald R. Waclaw,[1] Zegary J Allen II,[1] Sheila M. Bell,[1] Ferenc Erdélyi,[2] Gábor Szabó,[2] S. Steven Potter,[1] and Kenneth Campbell[1,*]
[1] Division of Developmental Biology
Department of Pediatrics
Children's Hospital Medical Center
University of Cincinnati College of Medicine
3333 Burnet Avenue
Cincinnati, Ohio 45229
[2] Department of Gene Technology
and Developmental Neurobiology
Institute of Experimental Medicine
Budapest
Hungary

Summary

The molecular mechanisms that regulate the production and diversity of olfactory bulb interneurons remain poorly understood. With the exception of the GABAergic/dopaminergic subtype in the glomerular layer, no information exists concerning the generation of the other subtypes. Here we show that the recently identified zinc finger transcription factor Sp8 is expressed in neurogenic regions, which give rise to olfactory bulb interneurons at embryonic and postnatal time points and remains expressed in the calretinin-expressing and GABAergic/nondopaminergic interneurons of the glomerular layer. Conditional inactivation of *Sp8* in the embryonic ventral telencephalon reveals a requirement for the normal generation of these interneuron subtypes. *Sp8* conditional mutants exhibit an increase in cell death within the lateral ganglionic eminence and rostral migratory stream. Moreover, mutant neuroblasts/interneurons are misspecified and display abnormal migration patterns in the olfactory bulb, indicating that Sp8 contributes to olfactory bulb interneuron diversity by regulating the survival, migration, and molecular specification of neuroblasts/interneurons.

Introduction

The subventricular zone (SVZ) of the lateral ventricular wall is a continuous source of neurons from early postnatal stages into adulthood (Luskin, 1993; Lois and Alvarez-Buylla, 1994). Progenitor cells in the SVZ generate migratory neuroblasts that travel in chains along the rostral migratory stream (RMS) to the olfactory bulb. There, they migrate radially and become postmitotic interneurons that populate the granule cell (GCL) and glomerular layers (GL). The adult SVZ has been shown to be heterogeneous based upon anatomical and ultrastructural characteristics (reviewed in Alvarez-Buylla and Garcia-Verdugo, 2002). Three main cell types have been proposed to exist in this progenitor region. The slowly-dividing stem cells (or B cells), which express GFAP and exhibit other astroglial characteristics, are precursors for the transit-amplifying progenitors (termed C cells), which are present in clusters scattered throughout the SVZ. The migratory neuroblasts (also called A cells), which express neuronal markers, such as β-III-tubulin or PSA-NCAM, are found in both the SVZ and RMS (Doetsch and Alvarez-Buylla, 1996). Little is known about the molecular control of this process; however, a number of transcription factors have recently been shown to label specific populations of these SVZ/RMS progenitors. For example, Dlx2 labels both transit-amplifying (i.e., C) cells and migratory neuroblasts (Doetsch et al., 2002), whereas Mash1 and Olig2 predominantly label populations of transit-amplifying cells (Hack et al., 2005; Parras et al., 2004). None of these factors remain expressed in the differentiated olfactory bulb interneurons that derive from the migrating neuroblasts; however, two other transcription factors have been identified in migrating neuroblasts that also mark differentiated interneurons, namely Er81 (Stenman et al., 2003a) and Pax6 (Hack et al., 2005; Kohwi et al., 2005). Pax6 has previously been shown to specifically mark the dopaminergic interneurons of the GL (Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005). Moreover, *Pax6* function is required for the neurogenesis of this cell type, at least at adult stages (Hack et al., 2005; Kohwi et al., 2005).

Dopaminergic interneurons make up only a portion of the olfactory bulb interneuron population. In fact, the diversity of this neuronal population has been rather underappreciated. At least three subtypes can be identified in the GL by the expression of calretinin, calbindin, or the GABA-synthesizing enzyme, glutamate decarboxylase (GAD) (Kosaka et al., 1995, 1998). The dopaminergic population comprises a subpopulation of the GABAergic interneurons (Kosaka et al., 1985, 1995, 1998). With the exception of Pax6 in the dopaminergic interneurons of the GL (Hack et al., 2005; Kohwi et al., 2005), the molecules that regulate olfactory bulb interneuron diversity are as yet unknown.

The lateral ganglionic eminence (LGE) is a known source of striatal projection neurons and olfactory bulb interneurons (Wichterle et al., 2001). A previous study (Stenman et al., 2003a) has suggested that, at embryonic stages, olfactory bulb interneurons arise from a progenitor domain in the dorsal region of the LGE (dLGE) (Yun et al., 2001). Moreover, this embryonic region has been hypothesized to give rise, at least in part, to the postnatal SVZ (Stenman et al., 2003a). In fact, all of the transcription factors that mark cell types in the postnatal SVZ and RMS (e.g., Dlx, Er81, Gsh2, and Pax6) are defining markers of the dLGE (Yun et al., 2001). Furthermore, mouse mutants for the homeobox gene *Gsh2* exhibit a severely reduced dLGE (Yun et al., 2001; Stenman et al., 2003a) along with significant reductions in olfactory bulb interneuron markers (Corbin et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001).

Recent studies have identified *Sp8*, a new member of the *Sp1* zinc finger transcription factor gene family (Bell

*Correspondence: kenneth.campbell@cchmc.org

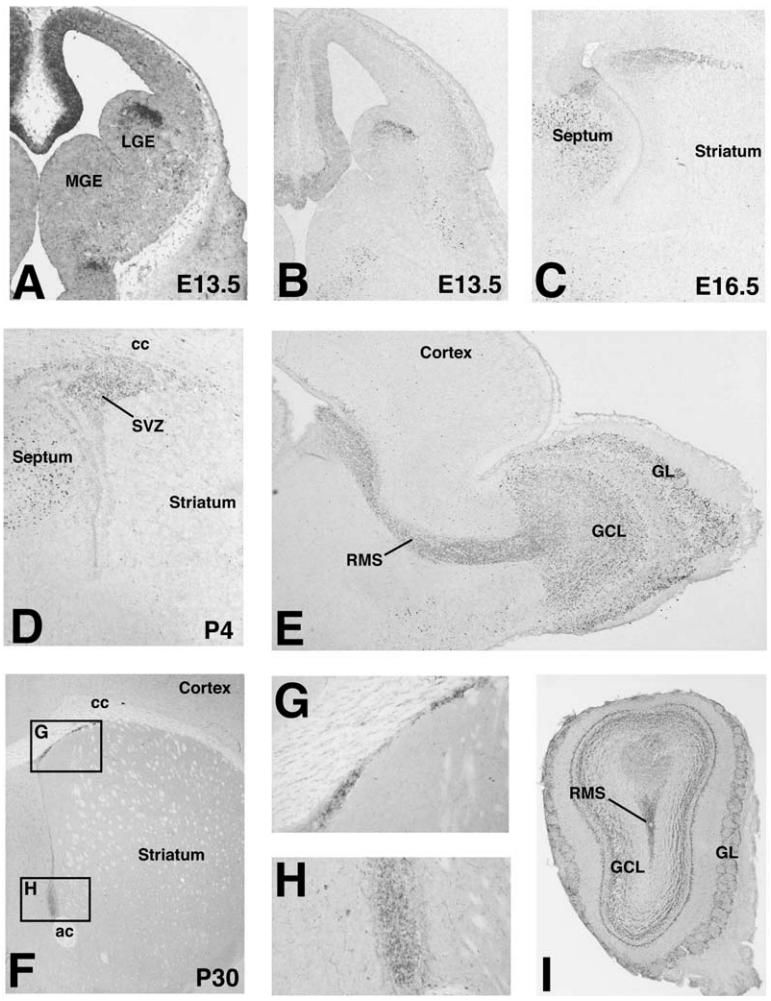

Figure 1. Sp8 Expression in the Embryonic dLGE, Postnatal SVZ, and Olfactory Bulb

(A–I) *In situ* hybridization (a) and immunohistochemistry (b–i) of *Sp8* and its protein product during embryonic and postnatal brain development. *Sp8* and its protein product are expressed in the dLGE at E13.5 (a and b) and E16.5 (c). Weak Sp8 expression is also observed in the dorsal medial telencephalon at E13.5 (b) and in cells of the lateral septum at E16.5 (c).

(D–E) The expression of Sp8 is observed at P4 in the forming SVZ in a coronal section (d) and along the entire RMS in a sagittal section (e). Sp8 expression persists in differentiating olfactory bulb interneurons of the granule cell layer (GCL) and glomerular layer (GL) (e).

(F–I) At P30, Sp8 expression is observed in the SVZ (f) at dorsal (g) and ventral (h) levels. In the olfactory bulb, Sp8 expression remains in the RMS and interneurons of the GCL and GL.

cc, corpus callosum.

et al., 2003; Treichel et al., 2003). In this study, we describe the expression pattern of Sp8 in neurogenic regions of the embryonic (dLGE) and postnatal (SVZ and RMS) brain. We also show that Sp8 marks specific populations of olfactory bulb interneurons, notably the calretinin and GABAergic/nondopaminergic populations of the GL. Germline *Sp8* mutants develop severe exencephaly, making the analysis of telencephalic structures impossible (Bell et al., 2003; Treichel et al., 2003). Utilizing a conditional knockout strategy, we have identified unique roles for *Sp8* in the generation of olfactory bulb interneuron diversity.

Results

Sp8 Expression in the dLGE

Precursor cells in the LGE are known to give rise to olfactory bulb interneurons and striatal projection neurons (Wichterle et al., 2001). Recent studies (Yun et al., 2001; Stenman et al., 2003a) have suggested that these two neuronal lineages derive from distinct compartments of the LGE, with progenitors in the dorsal region of the LGE (dLGE) giving rise to olfactory bulb interneurons while those in the ventral LGE produce striatal projection neurons. Here we describe the expression pattern of the recently discovered zinc finger transcription factor gene *Sp8* (Figure 1A; Bell et al., 2003; Treichel et al., 2003) and its protein product within the subventricular zone (SVZ) of the dLGE (Figures 1B and 1C). Sp8 expression comprises only the dorsal-most region of the *Dlx5/6* domain, as marked by EGFP expression in *Dlx5/6-cre-IRES-EGFP* (*Dlx5/6-CIE*) embryos (Figure 2A), which is reminiscent of the expression of Er81 in the dLGE (Stenman et al., 2003a). The expression of Sp8 initiates at around E10 in the SVZ of the dLGE and remains at high levels throughout embryogenesis. By E16.5, Sp8 is also observed in cells of the dorsolateral septum (Figure 1C). In addition, *Sp8* is expressed in the dorsomedial telencephalon (Figure 1A); however, its protein expression in this region is significantly lower than that observed in the dLGE (Figure 1B), despite the apparently similar levels of gene expression. This pallial expression is only observed at early stages of telencephalic development (i.e., between E10 and E14). Finally, a lateral stream of Sp8-positive cells is also observed from the dLGE toward the mantle region of the ventrolateral telencephalon (Figure 1B), a region previously suggested to contain derivatives of the dLGE (Yun et al., 2001).

The dLGE was originally defined as the region of the LGE that expresses high levels of *Gsh2*, *Pax6*, and *Er81* (Yun et al., 2001). Cells expressing Gsh2 and Pax6 protein show rather sharp boundaries in the ventricular zone

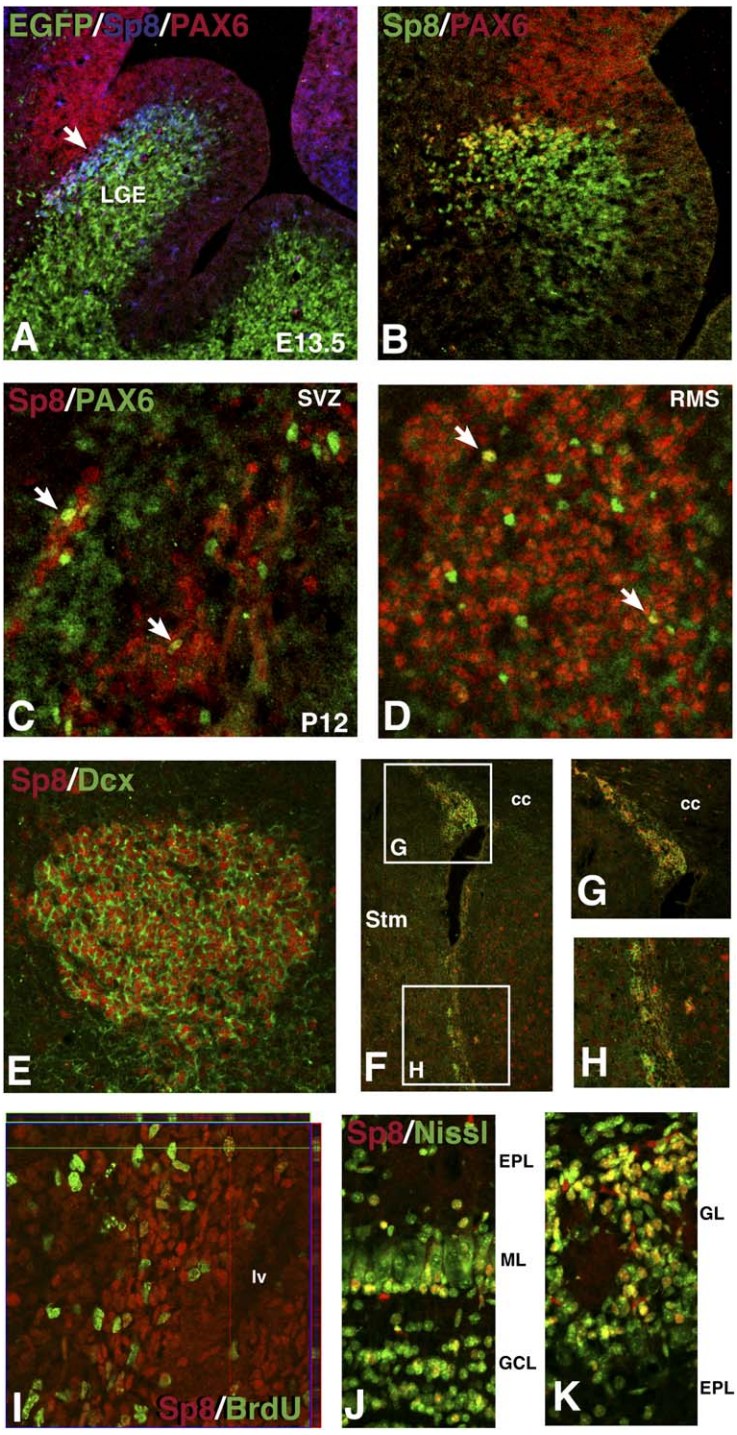

Figure 2. Cell-Type Expression of Sp8 in the Embryonic LGE and Postnatal SVZ/RMS

(A–B) Confocal images of the dLGE at E13.5. Triple immunostaining with Sp8, Pax6, and EGFP from the *Dlx5/6-CIE* transgene reveals Sp8 expression in the dorsal-most region of the *Dlx5/6* domain labeled with EGFP (a). Double staining with Pax6 and Sp8 shows evidence of heterogeneity in the SVZ of the dLGE with some colocalization. The majority of cells are Sp8-only and a minority of cells are Pax6-only (b).

(C–D) Heterogeneity is maintained in the postnatal (P12) SVZ (c) and RMS (d) with most cells being Sp8 positive and a much smaller population being Pax6-only or Sp8/Pax6 double-labeled cells (arrows).

(E–H) Sp8 is expressed in migrating neuroblasts of the postnatal RMS and SVZ. The vast majority of Sp8 positive cells co-express the migrating neuroblast marker Doublecortin (Dcx) in the RMS (e) and SVZ (f–h).

(I) Sp8 is expressed in some dividing cells in the postnatal SVZ. P4 mice were injected with 100mg/kg of BrdU and sacrificed 2 hr later. Confocal image of a cell co-expressing Sp8 and BrdU (Green/Red line intersection) shown as a representative from a Z-series.

(J–K) Sp8 positive neurons represent a subpopulation of interneurons in the granule cell layer (GCL) (j) and glomerular layer (GL) (k) as revealed by Sp8 staining (red) with a Nissl counterstain (green).

cc, corpus callosum; EPL, external plexiform layer; lv, lateral ventricle; ML, mitral cell layer; RMS, rostral migratory stream; Stm, striatum.

(VZ), with only a few cells overlaping at the pallio-subpallial boundary (Stenman et al., 2003b). Interestingly, Pax6 cells are found directly beneath this point of overlap in the dLGE SVZ (Stenman et al., 2003b). Double staining for Pax6 and Sp8 at E13.5 reveals a colocalization in some cells of the dLGE, with a majority of Sp8-only cells and a small minority of Pax6-only cells (Figure 2B). These findings suggest that already at embryonic stages the dLGE is a heterogeneous compartment with respect to molecular specification of cell types.

Sp8 Expression in the Postnatal SVZ, RMS, and Olfactory Bulb

It was previously suggested that the dLGE represents the predecessor of the postnatal SVZ (Stenman et al., 2003a). The expression pattern of Sp8 supports this notion, since it is found in cells that occupy the postnatal SVZ (Figure 1D) as well as the entire rostral migratory stream (RMS) (Figure 1E) at postnatal time points. Moreover, Sp8 expression persists in differentiating olfactory bulb interneurons in both the granule cell (GCL) and

glomerular layers (GL) (Figure 1E). At postnatal day (P)30 (i.e., in the more mature brain), Sp8 is expressed in cells of the SVZ (Figure 1F) along the entire lateral wall of the ventricle (Figures 1G and 1H) and remains expressed in cells of the RMS and differentiated olfactory bulb interneurons of both the GCL and GL (Figure 1I).

To further characterize the restricted expression pattern of Sp8 to specific cell types of the neurogenic regions (SVZ and RMS) and olfactory bulb interneurons, we utilized double-labeling immunofluorescence. The heterogeneity observed in the dLGE with respect to Sp8 and Pax6 expression appears to be maintained in the postnatal SVZ and RMS, with the majority of cells being Sp8 positive and smaller populations of Pax6-only and Sp8/Pax6 double-labeled cells (Figures 2C and 2D). Since Sp8 is expressed in the majority of cells within the SVZ and RMS, we hypothesized that many of these cells would be migrating neuroblasts (i.e., A cells). Indeed, at P12 most, if not all, Sp8-positive cells in the SVZ and RMS express the migrating neuroblast markers Doublecortin (Dcx) (Figure 2E–H) and PSA-NCAM (data not shown). Furthermore, following a short pulse with BrdU (2 hr), only a few Sp8-positive cells in the SVZ incorporated BrdU (Figure 2I). This is in line with the fact that some migrating neuroblasts remain in the cell cycle (Menezes et al., 1995; Doetsch et al., 1997). The majority of BrdU-positive cells, however, were Sp8 negative (Figure 2I). This suggests that most dividing progenitors of the SVZ and RMS do not express Sp8. In support of this notion, SVZ cells expressing Sp8 do not coexpress Mash1 (data not shown), which has recently been shown to mark at least some of the rapidly dividing transit-amplifying (i.e., C) cells (Parras et al., 2004).

To characterize the Sp8-positive neurons in the olfactory bulb, we first utilized a fluorescent Nissl counterstain. Sp8 expression in the olfactory bulb is found in many but not all interneurons in the GCL (Figure 2J) and GL (Figure 2K). Although Sp8-expressing cells were found in the mitral cell layer, they were small in diameter, similar to that of the granule cells that line the base of this layer (Figure 2J). Thus it appears that Sp8 marks only interneurons in the olfactory bulb consistent with a dLGE/SVZ origin. It is known there are at least three distinct populations of interneurons in the GL of the olfactory bulb that can be identified by the expression of the calcium-binding proteins, calbindin and calretinin, or the synthetic enzyme for GABA production, glutamic acid decarboxylase (GAD) (Kosaka et al., 1995, 1998). In addition, tyrosine hydroxylase (TH), which is the rate-limiting enzyme in dopamine production, labels a subpopulation of the GABAergic interneurons (Kosaka et al., 1985, 1995, 1998). With this information, we set out to fully characterize the Sp8-positive interneurons in the GL at P12. Interestingly, Sp8 is expressed in nearly all (97.6% ± 0.9%; 4001 cells analyzed, n = 3 animals) of the calretinin-positive cells in the GL (Figure 3A). Moreover, these double-positive cells represented 46.9% ± 0.3% of all Sp8-positive cells in the GL. In order to perform a cellular analysis of GAD and Sp8 colocalization, we used a GAD_{65}-GFP mouse line (De Marchis et al., 2004), which shows better cellular resolution than GAD immunostaining. Many GAD_{65}-positive cells in the olfactory bulb expressed Sp8 (Figure 3B). Indeed, within the GL, as many as 75.7% ± 2.0% of all GAD_{65} cells (4693 cells analyzed, n = 3 animals) coexpress Sp8. As was the case for calretinin interneurons, approximately half (56.9% ± 1.8%) of all Sp8 cells coexpressed GAD_{65}. Sp8 expression is only rarely observed within the TH population (Figure 3C; 13.6%; 2621 cells analyzed, n = 2 animals). Thus the majority of TH-positive cells likely correspond to the GAD-positive Sp8-negative cells present in the GL (Figure 3B). Even fewer of the calbindin-positive neurons in the GL (5.3%; 3515 cells analyzed, n = 2 animals) appear to colabel with Sp8 (Figure 3D). Furthermore, colocalization of Sp8 with each of these interneuron markers in the adult olfactory bulb yielded very similar results (data not shown). Finally, using the mitral and tufted cell marker Tbx21 (Yoshihara et al., 2005), we confirmed our observations from the Nissl stains that Sp8 is, in fact, not expressed by the projection neurons of the olfactory bulb (Figure 3C). Taken together, these results indicate that Sp8 is expressed in most migrating neuroblasts and remains expressed in subpopulations of mature olfactory bulb interneurons, namely the calretinin-positive cells and the nondopaminergic subpopulation of the GABAergic interneurons (see Figure 3E). In contrast to this, previous studies have reported that Pax6 is expressed in the vast majority of dopaminergic neurons of the GL and not in the calretinin- or calbindin-expressing interneurons (Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005). These findings suggest that the molecular heterogeneity observed with respect to Sp8 and Pax6 in the dLGE and postnatal SVZ/RMS may contribute to the development of distinct olfactory bulb interneuron subtypes.

Regulation of Sp8 Expression by *Gsh2*

From its earliest time of expression, Sp8 is restricted to the SVZ of the dLGE. Thus, factors expressed in the VZ of the LGE are likely to regulate its expression. The homeobox protein Gsh2 is expressed in a low ventral to high dorsal gradient in the LGE (Toresson et al., 2000a; Yun et al., 2001). The expression of Sp8 in the SVZ coincides precisely with the highest expression of Gsh2 in the LGE VZ (data not shown). Moreover, *Gsh2* gene function is required for Sp8 expression in the LGE (Figures 4B and 4G). Previous studies have shown that Pax6 is required for the repression of ventral telencephalic gene expression (e.g., *Gsh2*) within the dorsal telencephalic (i.e., pallial) VZ (Toresson et al., 2000a; Stoykova et al., 2000; Yun et al., 2001). Accordingly, in *Pax6* mutants, Sp8 expression expands dorsally into the SVZ of the pallium (Figure 4C). The results from these single mutants do not allow for the distinction between Gsh2 acting as a positive regulator of Sp8 expression or Pax6 functioning to repress its expression. By analyzing *Pax6;Gsh2* double mutants, we show that Sp8 expression in the dLGE depends on the expression of *Gsh2* in the dLGE VZ and is not simply repressed by the expansion of Pax6 in the *Gsh2* mutant (Figure 4D), as is the case for other ventral regulators such as Dlx proteins and Mash1 (Toresson et al., 2000a).

At birth, *Gsh2* mutants exhibit reductions in striatal projection neurons and olfactory bulb interneuron markers (Corbin et al., 2000; Toresson et al., 2000a; Toresson and Campbell, 2001; Yun et al., 2001; Stenman et al., 2003a). Stenman et al. (2003a) suggested that

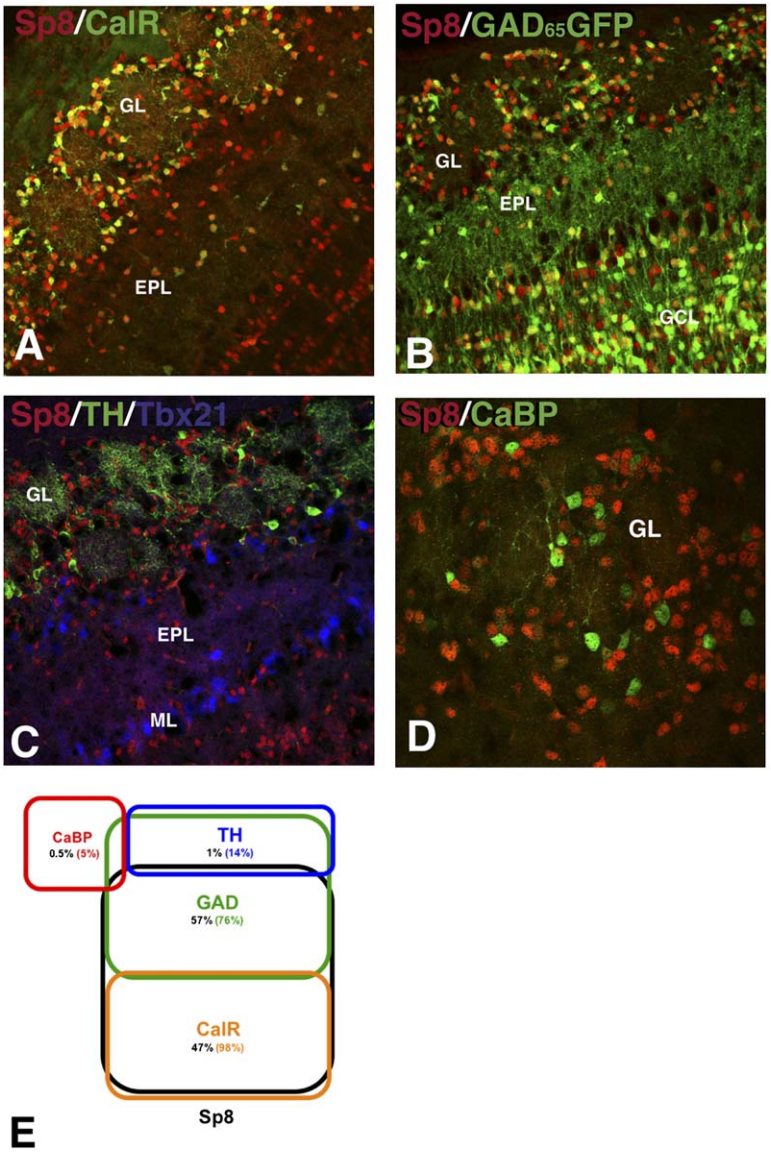

Figure 3. Sp8 Marks Specific Populations of Olfactory Bulb Interneurons in the Glomerular Layer

(A–D) Confocal images of postnatal olfactory bulbs at P12 (a,b,c,d). Sp8 expression is restricted to the calretinin (CalR) (a) and to a subset of the GAD-positive interneurons (b) of the granule cell layer (GCL) and glomerular layer (GL). GAD expression was observed by GFP staining from the $GAD_{65}GFP$ transgene. Sp8 is mostly excluded from the TH (c) and Calbindin (CaBP) (d) interneuron populations. Note the magnification in (d) is twice that of (a–c) and focused entirely on the GL. There was no overlap of Sp8 expression with the mitral/tufted projection neurons as marked by Tbx21 (c).

(E) Schematic diagram of olfactory bulb interneuron populations in the GL showing their respective proportions that express Sp8 (colored percentages). The percentage that each interneuron subtype represents of the total Sp8 population is indicated in black. The relative proportions of interneuron populations were modeled after Kosaka et al., 1998.

EPL, external plexiform layer; ML, mitral cell layer.

the reduction in olfactory bulb interneuron markers was due to a disruption in the formation of the dLGE. In support of this, we find that Sp8 expression remains severely reduced in the dLGE of *Gsh2* mutants at E18.5 (Compare Figure 4G to 4E). Moreover, Sp8-expressing interneurons are dramatically reduced in the *Gsh2* mutant olfactory bulb (Figure 4H) as compared to control (Figure 4F). These data indicate that Sp8 is downstream of Gsh2 and that the reduction of Sp8 expression in the *Gsh2* mutant leads, at least in part, to the reduced production of olfactory bulb interneurons in these mutants. Therefore, Sp8 may have a role in the normal generation of these neurons.

Conditional Inactivation of Sp8 Results in Olfactory Bulb Interneuron Defects

It has previously been shown that Sp8 is required for normal limb outgrowth and neural tube closure (Bell et al., 2003; Treichel et al., 2003). Germline deletion of *Sp8* results in severe exencephaly (compare Figure 5B to 5A), and therefore it is not possible to analyze its requirement in the developing brain, in particular during olfactory bulb interneuron generation from the dLGE and postnatal SVZ. To address this issue, we generated *Sp8* conditional mutants by mating mice with conditional *Sp8* alleles ($Sp8^{flox/flox}$) together with mice carrying a heterozygous *Sp8* conditional allele ($Sp8^{flox/+}$) and a *Dlx5/6-cre-IRES-EGFP* (*Dlx5/6-CIE*) transgene, which expresses cre recombinase specifically in the ventral telencephalon (Stenman et al., 2003a). The vast majority of *Sp8* conditional mutants ($Sp8^{flox/flox}$; *Dlx5/6-CIE*) did not exhibit exencephaly and developed a relatively normal looking brain with noticeably smaller olfactory bulbs than controls ($Sp8^{flox/+}$;*Dlx5/6-CIE*) (compare Figure 5D to 5C). In some cases, *Sp8* conditional mutant embryos developed exencephaly similar to *Sp8* null mutants. These embryos were not analyzed in this study. There was no detectable Sp8 expression in the dLGE of *Sp8* mutants at E13.5 (compare Figure 5F with 5E). This was also the case for the SVZ and RMS of conditional mutants at P4 and P12 (data not shown). In fact, the recombination of *Sp8* is complete by E11 (data not shown),

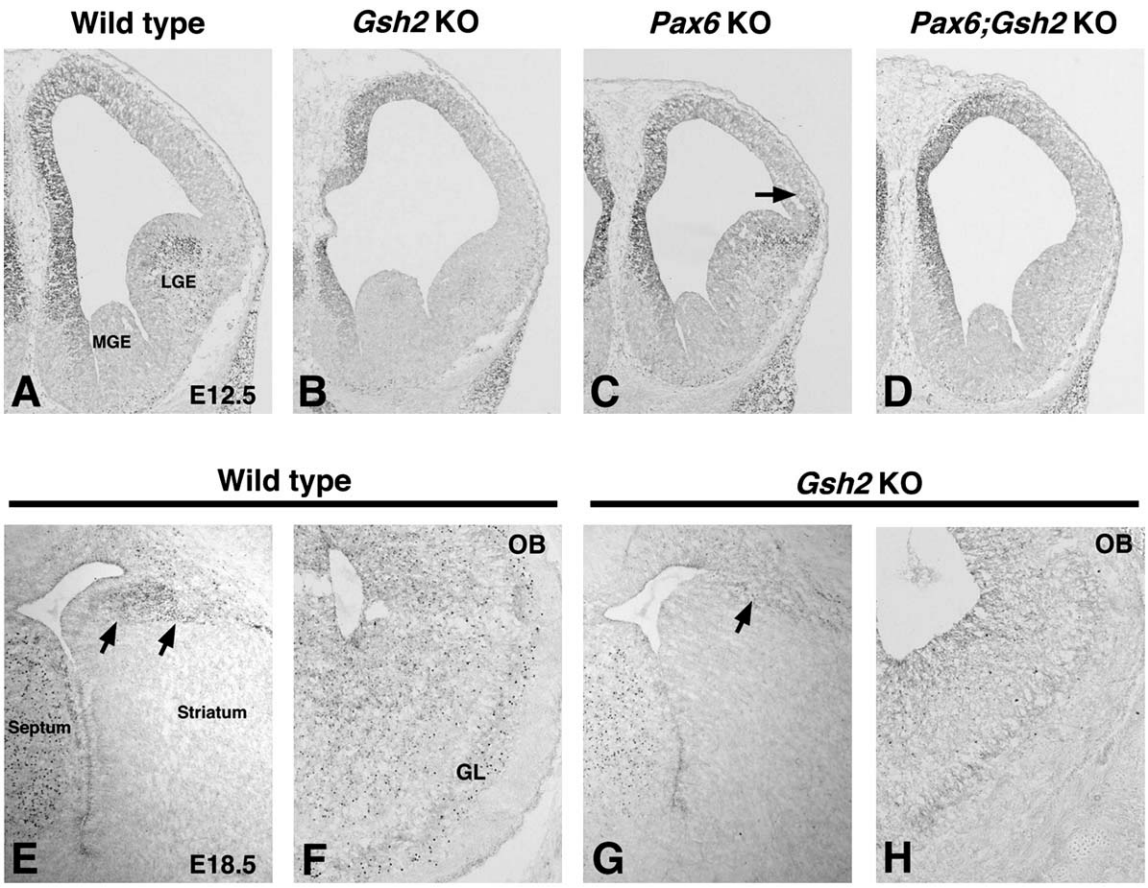

Figure 4. Sp8 Expression in the Embryonic dLGE and Olfactory Bulb Is Regulated by Gsh2

(A–D) Altered Sp8 expression detected by immunohistochemistry in *Gsh2*, *Pax6*, and *Pax6;Gsh2* mutants. At E12.5, Sp8 expression is absent in the dLGE of *Gsh2* mutants (compare b to a) and expands to the ventral pallium in *Pax6* mutants (arrow in c). *Pax;Gsh2* double mutants do not exhibit improved Sp8 expression (d), as is the case with other ventral telencephalic genes (e.g., DLX and Mash1). Therefore, the loss of Sp8 expression in *Gsh2* mutants is independent of the expansion of *Pax6* into the dLGE.

(E–H) Sp8 expression remains severely depleted in the E18.5 *Gsh2* mutant dLGE (compare arrow g to arrows in e) and olfactory bulb (compare h to f). Note the severe reduction of Sp8 positive neurons in the glomerular layer (GL) of the *Gsh2* mutant olfactory bulb.

OB, olfactory bulb.

which corresponds with the earliest time point that olfactory bulb interneurons are generated (Hinds, 1968). In addition, Sp8-expressing interneurons were completely lost in the olfactory bulb of *Sp8* conditional mutants at all stages examined (Figure 5H and data not shown). As would be predicted from the Sp8 expression pattern, the striatum (i.e., a ventral LGE derivative) of the conditional mutants did not show any gross defects (data not shown).

We next wanted to determine whether the loss of Sp8 has an effect on dLGE formation. Indeed, at E16. 5, cells expressing Er81 were specifically reduced in the dLGE of *Sp8* conditional mutants as compared to controls (Figures 6A and 6B). This was not the case for Pax6 cells in the dLGE, which were very similar in number between the control and conditional mutants (Figures 6C and 6D). Interestingly, the reduction in Er81 correlated well with an increase in cell death in the mutant LGE, as indicated by cleaved caspase-3 staining (Figures 6E and 6F). In the Sp8 mutant LGE, cleaved caspase-3-positive cells were increased 2.5 times over that seen in controls (control = 9.2 ± 4.0 caspase-3 cells/LGE; Sp8 mutant = 23.0 ± 1.4 caspase-3 cells/LGE; $p < 0.05$, $n = 3$). No obvious change in the number of dividing cells (i.e., phospho-Histone H3-positive cells) was observed in the mutant LGE. These results indicate that Sp8 is required for the survival of, at least, a subpopulation of Er81-positive dLGE cells, but not the Pax6 population.

Based on the expression of Sp8 in migrating neuroblasts and olfactory bulb interneurons, we set out to determine whether *Sp8* conditional mutants exhibited defects in their interneuron populations. We focused on the first 2 weeks after birth, when most of these interneurons are being generated (Hinds, 1968). We made use of the EGFP in the *Dlx5/6-CIE* mice, which is expressed in migrating neuroblasts and differentiating olfactory bulb interneurons of the GCL and GL around birth (Stenman et al., 2003a; Figure 6G). Interestingly, there is a noticeable decrease in EGFP-expressing cells in the *Sp8* conditional mutant olfactory bulb, particularly in the GL (Figure 6H). Consistent with this result and the E16.5 data described above, the E18.5 mutant olfactory bulbs exhibit a severe reduction in Er81-expressing cells predominantly within the forming GL (compare Figure 6I with 6J). These findings are very similar to those previously reported in *Gsh2* mutants (Stenman et al.,

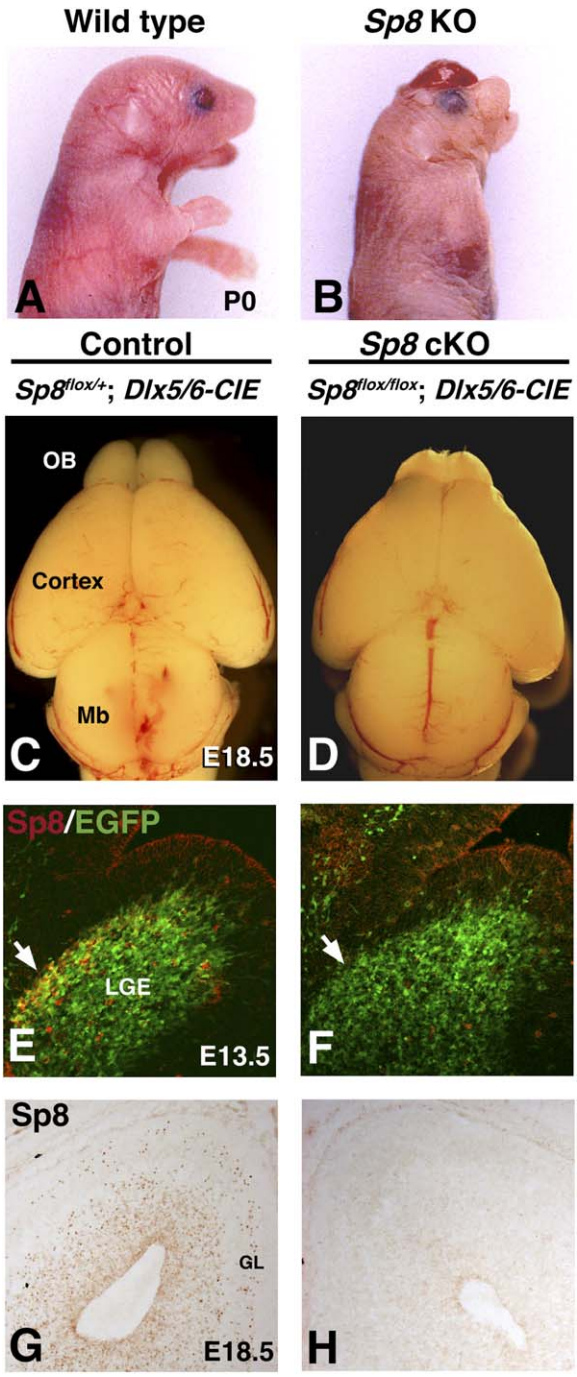

Figure 5. Conditional Inactivation of *Sp8* in the Ventral Telencephalon

(A–D) Germline *Sp8* mutants exhibit severe exencephaly at P0 (compare b to a). Utilizing the ventral telencephalon specific *Dlx5/6-cre-IRES-EGFP* (*Dlx5/6-CIE*) mouse, conditional *Sp8* mutants (cKO, $Sp8^{flox/flox}$;*Dlx5/6-CIE*) were created that develop a relatively normal looking brain with consistently smaller olfactory bulbs(OB, d) than controls ($Sp8^{flox/+}$;*Dlx5/6-CIE*) (c) at E18.5.

(E–H) *Sp8* conditional mutants do not express Sp8 in the dLGE at E13.5 (compare arrows in f and e). Sp8 expression is also lost in olfactory bulb interneurons (E18.5, compare h to g).

GL, glomerular layer; Mb, midbrain.

2003a). A reduction in Er81-positive cells within the *Sp8* conditional mutant olfactory bulb is also observed at P12 (compare Figure 7H with 7G), when the peak in neurogenesis of olfactory bulb interneurons has occurred (Hinds, 1968). At this stage it is clear that Er81-positive cells are reduced in both the GCL and GL (Figure 7H).

By P12, when the mature cytoarchitecture is established in the control olfactory bulb (Figure 7A), the *Sp8* conditional mutant olfactory bulb appears very disorganized (Figure 7B). In the mutants, no clear distinction exists between the mitral cell layer and the GCL (i.e., no internal plexiform layer). Moreover, the external plexiform layer is reduced in size. In the GL, glomeruli appear to be stacked on top of each other, in some cases three to four glomeruli deep. To determine whether the defects in the *Sp8* conditional mutant olfactory bulb include the locally generated projection neurons, we used markers of the mitral and tufted cells, Tbr1 (Bulfone et al., 1998) and Tbx21 (Yoshihara et al., 2005). No reduction in Tbr1-expressing cells was observed in the conditional mutant olfactory bulb at any stage after birth (Figure 7F and data not shown). In fact, in most cases the density appeared to be increased, perhaps due to the reduced size of the olfactory bulb. This was also the case for Tbx21-expressing cells. This marker clearly identifies mitral cells, which typically form a single cell layer (Figure 7C). In the *Sp8* conditional mutants, Tbx21-positive cells appear somewhat scattered, leading to a widening of the mitral cell layer (Figure 7D). This is also evident in the Nissl stains (Figure 7B). Thus, conditional inactivation of *Sp8* in the dLGE and postnatal SVZ not only leads to reduced numbers of olfactory bulb interneurons but also to an altered cytoarchitecture of the mutant olfactory bulb.

To determine the requirement of Sp8 for the generation and/or survival of distinct interneuron subtypes, we analyzed the expression of GAD67, calretinin, calbindin, and TH within the first 2 weeks after birth. *Sp8* conditional mutants exhibited severe reductions in GAD67-expressing interneurons of both the forming GCL and GL around birth (Compare 6K with 6L). A significant reduction in GAD67 expression is also evident at P12 (Figure 7J). At this later stage, GAD67 stains neuropil strongly, making cellular quantification difficult (see inset in Figure 7I). For this reason, we counted the GAD67 cells at E18.5, when cell bodies were easy to identify (Figures 6K and 6L). At this stage, the *Sp8* conditional mutants exhibited a 59% and 65% reduction in GAD67-positive cells in the GCL and GL, respectively (GCL, 72.8 ± 2.7 versus 29.7 ± 2.7 cells/section, $p < 0.001$; GL, 59.5 ± 1.4 versus 20.7 ± 1.9 cells/section, $p < 0.0001$; control n = 3, mutant n = 4). In addition to GAD67, interneurons expressing calretinin were also reduced in the *Sp8* conditional mutant olfactory bulb at both P4 (Figure 8H) and P12 (Figure 7L), particularly in the GL. At P12, the number of calretinin neurons in the GL was reduced by 50% from that in the control (59.3 ± 0.8 versus 29.4 ± 1.4 cells/field, $p < 0.0001$; n = 4). Unlike the GAD and calretinin populations, calbindin- and TH-expressing interneurons were less affected in the *Sp8* conditional mutants. At P12, the number of calbindin cells in the mutant GL (Figure 7N) is very similar to the control (Figure 7M). In fact, there is only a 13% reduction in the mutant olfactory bulb from the numbers in the control (24.4 ± 0.3 versus 21.3 ± 0.9 cells/field, $p < 0.03$; n = 4). A slightly greater reduction (19%, 28.4 ± 1.4 versus 23.0 ± 1.0 cells/field, $p < 0.03$; n = 4) is seen for

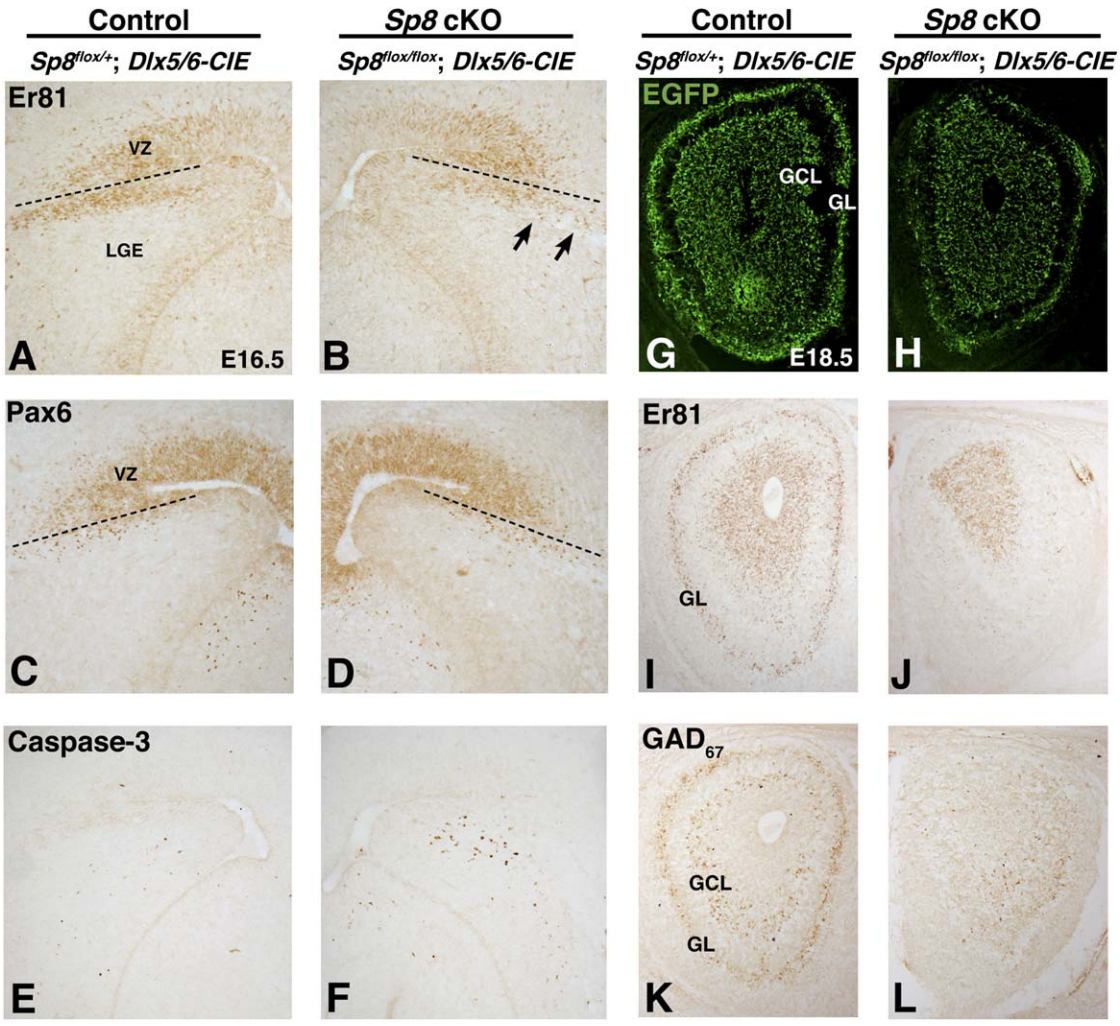

Figure 6. *Sp8* Conditional Mutants Exhibit Embryonic Defects in the dLGE and Olfactory Bulb Interneuron Generation

(A–F) Representative coronal sections of E16.5 control (a,c,e) and *Sp8* conditional mutant (b,d,f) LGEs. The Er81 expression domain in the dLGE is reduced in *Sp8* conditional mutants (arrows in b) compared to control (a). The dashed lines indicate the pallio-subpallial boundary. Note the similar expression of Er81 in the pallial VZ (i.e., above the dashed line) of the conditional mutant (b) and control (a). Pax6 expression in the dLGE is unchanged in the *Sp8* conditional mutants (compare d to c). Apoptotic cells, as marked by cleaved Caspase 3 expression, are significantly increased in the *Sp8* conditional mutant LGE (f) compared to control LGE (e).

(G–L) Representative coronal sections of E18.5 control (g,i,k) and *Sp8* conditional mutant (cKO) (h,j,l) olfactory bulbs. *Sp8* conditional mutants have severe reductions in embryonic olfactory bulb interneurons. There is a noticeable decrease in EGFP positive interneurons from the *Dlx5/6-CIE* transgene in the *Sp8* mutant (h) compared to control (g). Er81 expression is almost completely lost at this stage in the forming glomerular layer of the *Sp8* conditional mutant (j) compared to control (i). Interneurons in both the forming granule cell layer (GCL) and glomerular layer (GL) that express GAD67 are significantly decreased in the *Sp8* mutant (l) compared to control (k).

TH-positive GL cells in the *Sp8* conditional mutants (compare Figure 7P with 7O). Together these results indicate that interneurons are specifically affected in the *Sp8* conditional mutants and that the most severely affected populations are those that normally maintain expression of Sp8 as differentiated neurons (i.e., the calretinin and GABAergic/nondopaminergic populations).

Altered Pax6 Expression in the RMS and Olfactory Bulb of *Sp8* Conditional Mutants

In addition to the observed decreases of interneuron subtypes, migratory abnormalities were also observed in the RMS and olfactory bulb of *Sp8* conditional mutants. First, the number of cells expressing Pax6 in the RMS, at olfactory bulb levels, is dramatically increased at perinatal stages (e.g., P4, Figure 8B). This increase remains evident even at P12 (Figure 8D). We quantified the difference at this stage and found that the RMS, at olfactory bulb levels, of *Sp8* conditional mutants contains 2.6 times more Pax6 cells than the controls (64.3 ± 2.0 versus 25.1 ± 0.9 cells/section, $p < 0.0001$; $n = 4$). The majority of these Pax6 cells double labeled with Dcx (Figure 8F), as was the case in controls (Figure 8E), suggesting that they are migrating neuroblasts. Notably, at SVZ levels the number of Pax6-positive cells in control and *Sp8* conditional mutants was not statistically different (29.3 ± 0.6 versus 24.8 ± 2.9, $p = 0.18$; $n = 4$). In addition, starting at E18.5, we found clumps of Pax6-positive cells in the GCL of the *Sp8* conditional mutant olfactory bulbs, rather than in the GL, as was the case

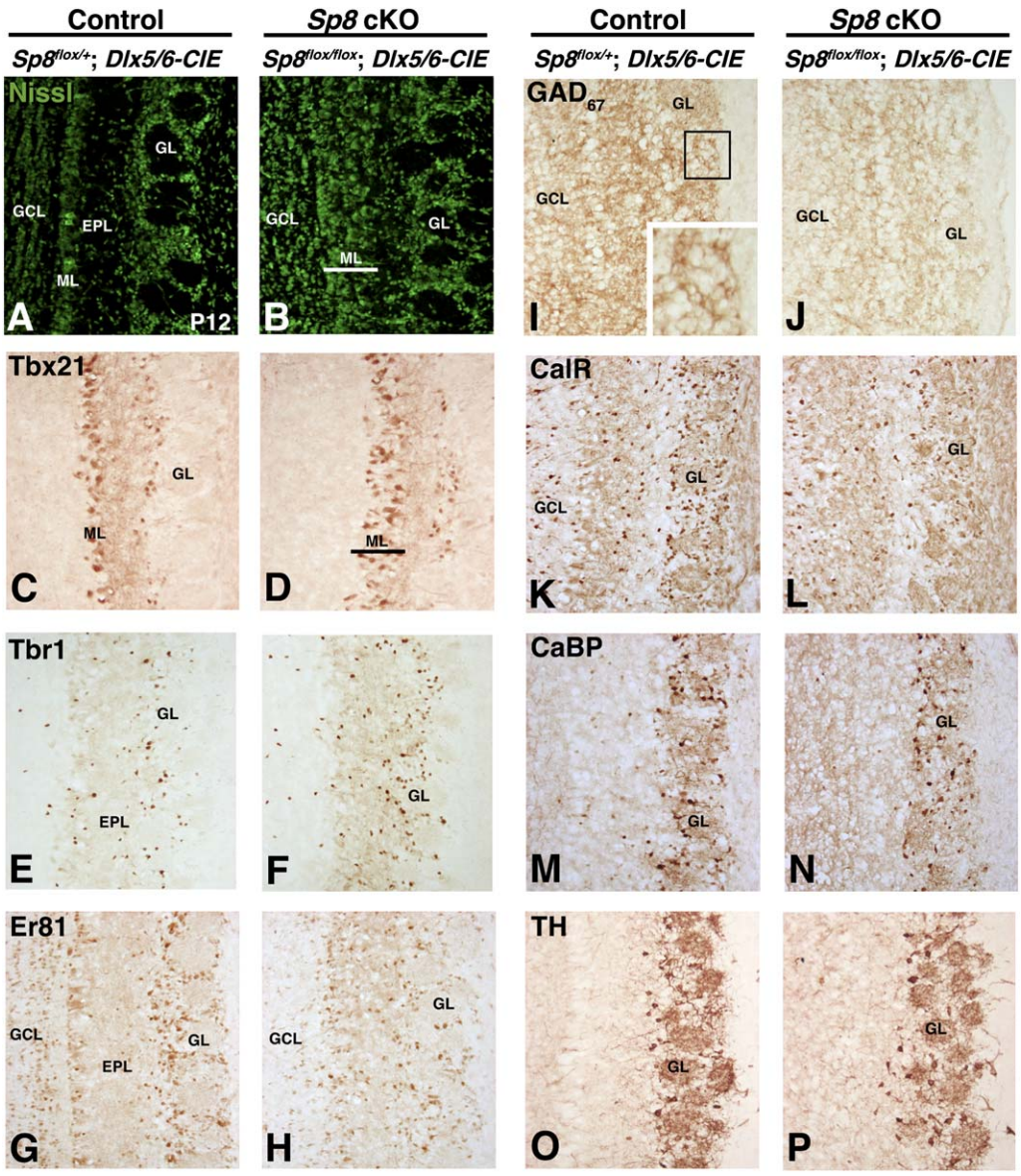

Figure 7. *Sp8* Conditional Mutants Exhibit Subtype-Specific Defects in Generation of Olfactory Bulb Interneurons

(A–N) Representative coronal sections of P12 control (a,c,e,g,i,k,m,o) and *Sp8* conditional mutant (cKO) (b,d,f,h,j,l,n,p) olfactory bulbs. The cytoarchitecture revealed by Nissl stain of *Sp8* mutant olfactory bulbs is severely disorganized with no clear distinction between the mitral cell layer (ML) and granule cell layer (GCL) (compare b to a). There is no reduction in locally born projection neurons labeled with Tbx21 in mitral cells (compare d to c) and Tbr-1 in tufted cells (compare f to e) in the conditional mutant olfactory bulb. Note the widened expression domain of Tbx-21 in the mitral cell layer of the *Sp8* mutant (bar in d). This is consistent with the altered cytoarchitecture observed with the Nissl stain (bar in b). Olfactory bulb interneurons as marked by Er81 are not normally generated in the *Sp8* conditional mutant GCL and glomerular layer (GL) (compare h to g). Specific populations of olfactory bulb interneurons in the GL are severely affected in the *Sp8* conditional mutants. GAD67 (compare j to i) and calretinin (CalR) (compare l to k) staining is noticeably depleted in the GL of the *Sp8* mutant olfactory bulb, where as calbindin (CaBP) (compare n to m) and tyrosine hydroxylase (TH) (compare p to o) positive neurons appear to be less affected. Inset in i shows high power of the boxed region. Note the strong neuropil staining of GAD67 in the GL making cell body delineation difficult.
EPL, external plexiform layer.

in controls (see Figures S1A and S1B in the Supplemental Data available online).

Similar to the findings with Pax6, we found clumps of calretinin cells in the GCL of the *Sp8* mutants at perinatal stages (Figure 8H) and an accumulation of calretinin cells in the RMS, at olfactory bulb levels, by P12 (Figure 8J). Interestingly, many of these calretinin-positive cells colocalized Pax6, both within the clumps in the GCL (Figure 8K) as well as in the cells of the RMS (Figure 8L). As previously shown, Pax6 normally does not colocalize with calretinin in olfactory bulb interneurons (Dellovade et al., 1998; Hack et al., 2005), indicating that many of the calretinin-positive cells generated in the *Sp8* mutant are misspecified. We also observed clumps of calbindin and GAD67 cells within the *Sp8* mutant RMS at P12 (Figure S2); however, these findings were

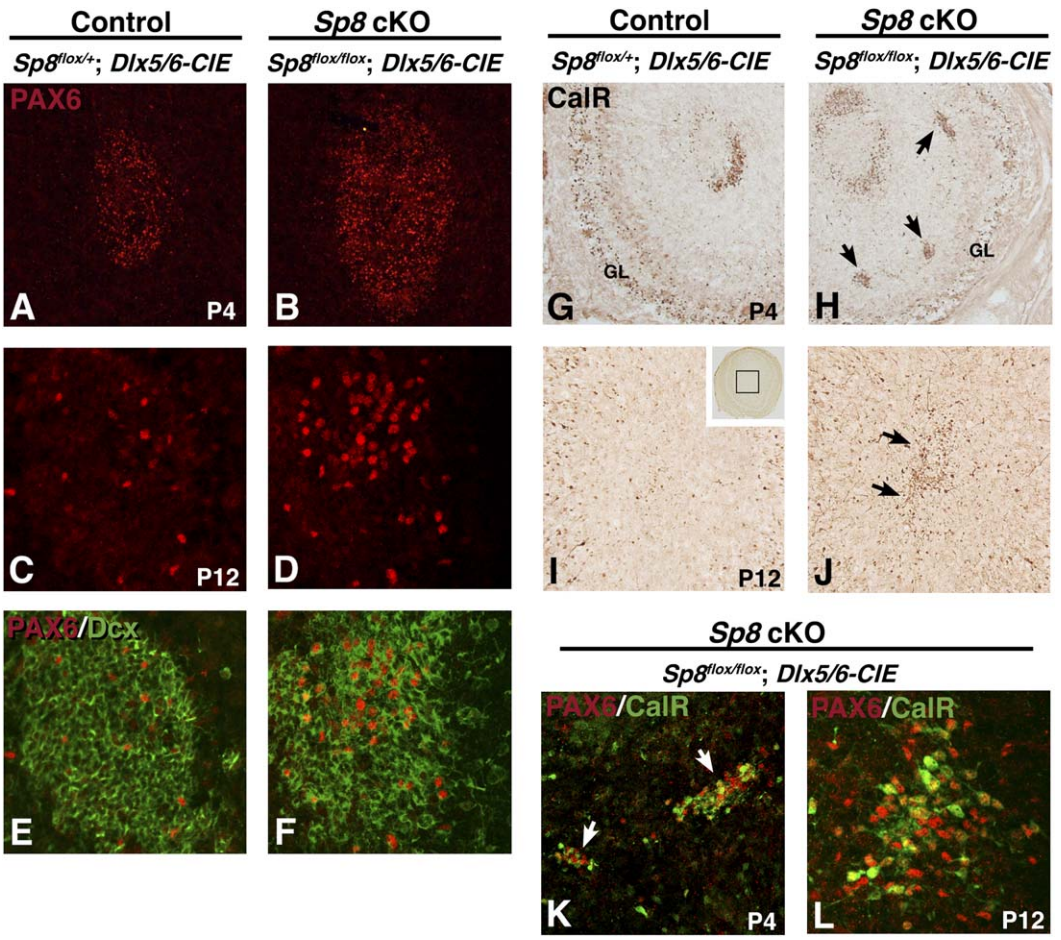

Figure 8. Altered Expression of Pax6 and Calretinin in the Olfactory Bulbs of *Sp8* Conditional Mutants

(A–F) The number of Pax6 positive cells in the RMS is increased in the *Sp8* conditional mutant at P4 (b) and P12 (d) compared to control (a and c), as detected by fluorescent immunostaining. The majority of Pax6 cells in the *Sp8* cKO RMS were double labeled with Dcx (f), indicating they were migrating neuroblasts.

(G–L) Abnormal calretinin (CalR) expression observed in the *Sp8* conditional mutant (cKO). Clusters of calretinin neurons were detected in the developing granule cell layer (GCL) of the P4 *Sp8* conditional mutant olfactory bulb (h). These clusters were not observed in control olfactory bulbs (g). Many of these abnormal calretinin neurons in the *Sp8* conditional mutant olfactory bulbs aberrantly co-expressed Pax6 (k). Box in inset (i) reveals location of high power images from the RMS at olfactory bulb levels in P12 control (i) and *Sp8* conditional mutants (j). Increased expression of calretinin (CalR) (j) was also observed in the RMS of *Sp8* mutant olfactory bulbs as compared to the control RMS (i). The ectopic calretinin expression in the *Sp8* conditional mutant RMS was throughout the rostrocaudal extent of the olfactory bulb and similar to the clumps in the GCL (h), many of these cells aberrantly co-expressed Pax6 (l).

much less frequent than those for the calretinin cells. We did not attempt to colocalize Pax6 in the clumps of GAD-positive cells, since it is normally the case that a significant population of these cells coexpress Pax6 (presumably the dopaminergic/GABAergic subpopulation) (Hack et al., 2005; Kohwi et al., 2005).

Consistent with the idea that at least some of the neuroblasts/interneurons are inappropriately specified, we found that cell death, as assayed by TUNEL staining, was increased more than two times in the RMS and olfactory bulb of *Sp8* conditional mutants at P12, as compared to the control (45.0 ± 8.9 versus 21.3 ± 4.6 cells per animal, $p < 0.05$; $n = 4$). Taken together these findings suggest that Sp8 contributes to the diversity of olfactory bulb interneurons by regulating the survival, migration, and molecular specification of migrating neuroblasts/interneurons.

Discussion

Using a conditional mutagenesis strategy, we have uncovered a crucial requirement for Sp8 in the regulation of olfactory bulb interneuron development. Sp8 is expressed in most neuroblasts of the RMS and remains expressed in each of the calretinin and GABAergic/non-dopaminergic interneuron subtypes. The present results demonstrate that these two interneuron populations are most dependent on Sp8 function for their normal development and/or survival. Moreover, at least the neuroblasts, which generate calretinin interneurons require Sp8 for their correct specification. In its absence, these cells misexpress Pax6 and display abnormal migratory behaviors. Together with the recent work on the role of Pax6 in the dopaminergic interneurons (Hack et al., 2005; Kohwi et al., 2005), these findings contribute

significantly to an emerging model for the molecular specification of distinct olfactory bulb interneuron subtypes.

The expression of Sp8 shown here in the embryonic and postnatal telencephalon lends further support to the notion that the dLGE gives rise to the postnatal SVZ, as previously suggested (Stenman et al., 2003a). The fact that Pax6 is expressed in the postnatal SVZ and RMS (Hack et al., 2005; Kohwi et al., 2005) might suggest that these neurogenic regions also derive from a more dorsal telencephalic (i.e., pallial) region. However, Yun et al. (2001) used high *Pax6* expression as one of the defining features of the dLGE. Indeed, Pax6 expressing cells in the SVZ of the embryonic telencephalon derive from the pallio-subpallial boundary (Yun et al., 2001), precisely where Pax6 and Gsh2 are co-expressed over a few cell diameters (Stenman et al., 2003b). The present results demonstrate that these Pax6 cells are largely confined to the dLGE and many of them even co-express Sp8. It is feasible therefore that the cells which give rise to Pax6 cells in the postnatal SVZ and RMS were seeded from the dLGE. Thus the diversity of cell types that constitute the postnatal SVZ/RMS may derive largely from the VZ of the dLGE. This embryonic progenitor domain has also been proposed to give rise to structures in the ventrolateral telencephalon (including the piriform cortex and certain amygdalar nuclei) (Yun et al., 2001; Puelles et al., 2000). In accordance with this notion, Sp8 expressing cells are also seen in a lateral stream from the dLGE to the ventrolateral telencephalon (see e.g., Figure 1B). Thus, the postnatal SVZ likely represents the major structure that derives from the dLGE but not its sole derivative.

The interneurons that arise from the dLGE or SVZ/RMS migrate as neuroblasts rostrally to populate the GCL and GL of the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Pencea and Luskin, 2003). Although many of the interneurons in both of these layers are GABAergic, not all are. In fact, within the GL, the GABAergic interneurons have been suggested to comprise only about 20% of all cells (Kosaka et al., 1995, 1998). Of the GABAergic interneurons, up to half are likely to be dopaminergic as well (Kosaka et al., 1985, 1995, 1998). Thus, there are a number of other periglomerular cells for which the neurotransmitter is not known. However, these cells can be marked by the expression of the calcium binding proteins calretinin or calbindin. For the most part, these are distinct non-overlapping populations (Kosaka et al., 1995; 1998). While most migrating neuroblasts appear to express Sp8 in the RMS, only two of the interneuron subtypes express it as mature neurons in the GL, specifically the calretinin and GABAergic/non-dopmainergic subtypes. Within the GCL, many but not all GABAergic neurons express Sp8. It seems likely, therefore, that Sp8 is down-regulated in, at least, some of the mature interneurons. Pax6 is expressed in fewer neuroblasts within the RMS than Sp8 and remains expressed primarily in the dopaminergic interneurons of the GL (Hack et al., 2005; Kohwi et al., 2005). This factor has also been suggested to down-regulate in some differentiating interneurons, specifically those in the GCL (Hack et al., 2005). Similar to Sp8, the ETS transcription factor Er81 is expressed in large numbers of migrating neuroblasts as well as differentiated interneurons of both the GCL and GL (Stenman et al., 2003a). Due to technical limitations with same species antibodies we have not, as yet, been able to determine the extent of overlap between Sp8 and Er81 in this system. In any case, the expression of Sp8 and Pax6 in distinct subtypes of interneurons provides, for the first time, a framework for establishing a molecular code of olfactory bulb interneuron specification. Understanding this molecular heterogeneity will ultimately prove quite useful in elucidating the molecular mechanisms underlying the generation of distinct interneuron subtypes.

In the *Sp8* conditional mutants, we observe severe reductions in both calretinin and GABAergic interneuron subtypes. However, the effect on the calbindin and dopaminergic populations is considerably milder. Given the noticeable reduction in the size of the olfactory bulb within the conditional mutants it is not surprising that all populations are significantly reduced in numbers. It is interesting though that the most severe reductions are seen in the populations which continue to express Sp8 as mature neurons. This fact suggests that maintained expression of Sp8 is necessary for these cell types to fully differentiate, migrate and/or survive. Indeed, we observed an increase in cell death within the dLGE at late embryonic stages as well as in the postnatal RMS and olfactory bulb of the conditional mutants. It seems likely that the calretinin and GABAergic/non-dopaminergic populations were impacted by this cell death more so than the dopaminergic and calbindin interneurons. In addition to the increased cell death, calretinin cells displayed abnormal migration patterns in the Sp8 conditional mutants. At perinatal time-points they were found in clumps within the GCL. By P12, calretinin-positive cells were also found in clumps along the rostro-caudal extent of the RMS at olfactory bulb levels.

Until now, the neuroblasts (or A cells) of the SVZ/RMS have mainly been described as a homogeneous population with respect to molecular markers (i.e., all express Dcx, β-III-tubulin and PSA-NCAM). Recently, Pax6 has been shown to mark a subset of migratory neuroblasts (Hack et al., 2005; Kohwi et al., 2005). Although Sp8 is expressed by most neuroblasts, the expression of Pax6 either alone or together with Sp8 in these cells indicates that migrating neuroblasts are heterogeneous. In support of this notion, the GAD_{65} gene is expressed in, at least, a subset of migrating neuroblasts, none of which actually express the protein (De Marchis et al., 2004). Interestingly, although Sp8 is found in most of the GAD_{65}-GFP-positive neuroblasts of the RMS, many Sp8-only cells as well as a few GAD_{65}-GFP-only cells are also present (data not shown). These results are reminiscent of our findings in the differentiated olfactory bulb interneurons with respect to Sp8 and GAD_{65} expression. Thus the migrating neuroblasts which give rise to GABAergic interneurons are likely to be specified already in the RMS and possibly even in the SVZ. In this respect, the GAD_{65}-GFP -positive Sp8 negative neuroblasts could represent the GABAergic/dopaminergic phenotype known to express Pax6 (Hack et al., 2005; Kohwi et al., 2005). In the *Sp8* conditional mutants, we observed a large increase in the number of neuroblasts expressing Pax6 in the RMS. Additionally, Pax6-positive cells were found clumped in the GCL of the perinatal olfactory bulb. Interestingly, many of these cells

aberrantly expressed calretinin (Dellovade et al., 1998; Hack et al., 2005). Therefore, it seems possible that in the neuroblasts destined to form calretinin interneurons, Sp8 functions as a negative regulator of Pax6 expression. In zebrafish, Sp8 has been suggested to act as an activator (Penberthy et al., 2004), however, Sp proteins are known to exhibit both repressor and activator activities (Zhao and Meng, 2005). Thus it remains unclear whether the regulation of Pax6 by Sp8 in these neuroblasts is direct or indirect (e.g., by activation of a repressor). Nevertheless, in the absence of *Sp8*, Pax6 may interfere with the calretinin neuron's ability to migrate correctly and/or survive.

Neuroblasts normally migrate through the RMS using chain migration (Wichterle et al., 1997) and when they reach the olfactory bulb they must switch to radial migration in order to reach their final position in the GCL or GL. The conditional mutant neuroblasts do not seem to be severely impaired in chain migration since many interneurons (particularly the calbindin and dopaminergic subtypes) do reach the mutant olfactory bulb. However, by P12, we found clusters of neurons within the RMS, specifically at olfactory bulb levels, that express markers of differentiated interneurons such as calretinin as well as calbindin and GAD (although less expansive on a rostral caudal level than calretinin neurons). In addition, at early perinatal stages we observed clusters of calretinin neurons in the forming GCL. Radial glial morphology, as marked by Nestin and GLAST, appears relatively normal in most areas of the conditional mutant olfactory bulb (Figures S1C and S1D), suggesting that the observed migration defect is likely to be cell autonomous. Indeed, aberrant Pax6 expression (discussed above) may impair the ability of the *Sp8* conditional mutant neuroblasts to undergo normal radial migration. Moreover, this effect may be sufficient to initiate a cell death program.

A recent study (Kawakami et al., 2004), has identified another new member of the *Sp1* gene family, *Sp9*. *Sp9* shares significant sequence homology with *Sp8* (Kawakami et al., 2004) and also exhibits a partially overlapping expression pattern in both the embryonic and postnatal telencephalon (unpublished observations). Indeed, we have observed expression of *Sp9* in the telencephalon of *Sp8* conditional mutants (data not shown). Thus it is possible that *Sp9* may partially compensate for *Sp8*. It will be interesting to determine the consequences for olfactory bulb interneurons after inactivating both *Sp8* and *Sp9*.

In addition to the impaired generation of interneurons in the *Sp8* conditional mutants, the general cytoarchitecture of the mutant olfactory bulb was also severely disrupted. The conditional mutants lacked the normal laminar structure typical of the control olfactory bulb. These mutants did not exhibit distinct internal plexiform or external plexiform layers. Furthermore, the mitral cell layer, which normally exists as a single cell layer, was disorganized. The Arx homeobox protein is expressed in migrating neuroblasts and olfactory bulb interneurons but like Sp8 is not found in the projection neurons (Yoshihara et al., 2005). Interestingly, similar cytoarchitectural alterations to those shown here were observed in the *Arx* mutants, which also exhibit severe reductions in olfactory bulb interneurons (Yoshihara et al., 2005). Together with the present data, these results indicate that interneurons contribute to the cytoarchitectural organization of the forming olfactory bulb. This is somewhat surprising since these neurons are predominantly generated at later time-points than the locally derived projection neurons (Hinds, 1968). It may be therefore, that the interneurons generated at embryonic stages, which comprise only a fraction of those generated in the first few weeks after birth (Hinds, 1968), play a crucial role in organizing the cytoarchitecture of the olfactory bulb. Future studies in which *Sp8* is intact embryonically and conditionally inactivated at postnatal stages may help to address this issue.

In summary, we show here that the zinc finger transcription factor Sp8, represents a novel marker of migrating neuroblasts as well as subtypes of olfactory bulb interneurons, namely the calretinin-expressing and GABAergic/non-dopaminergic populations of the GL. Not only is Sp8 expressed in these interneuron subtypes, it is also required for their normal generation. In particular, our results demonstrate that Sp8 is required for the survival, migration and correct specification of these neuroblast/interneuron subtypes and thereby contributes to the diversity of olfactory bulb interneurons.

Experimental Procedures

Animals

Gsh2 (Szucsik et al., 1997) and *Pax6 (Small eye, Sey)* (Hill et al., 1991) mouse embryos and adults were genotyped as described previously (Toresson et al., 2000a). *$GAD_{65}GFP$* mice were described in De Marchis et al. (2004). *$Sp8^{Flox/+}$* (Bell et al., 2003) mouse embryos and adults were genotyped by PCR with the following primers: Spd1-TCCTCCCACGAGTGTAATGCTCAG and Spd2- GCGTTCTTTCCC CCAACTTC. The *Sp8* wild-type allele results in a 375 bp product, where as the *Sp8* flox allele results in a 450 bp product. *Dlx5/6-Cre-iresEGFP* (*Dlx5/6-CIE*) mice were genotyped as previously described (Stenman et al., 2003a). *Sp8* conditional mutants were obtained from crossing double heterozygous males (*$Sp8^{Flox/+}$*; *Dlx5/6-CIE*) with *Sp8* homozygous flox (*$Sp8^{Flox/flox}$*) females. For staging of embryos, the morning of vaginal plug detection was designated embryonic day (E) 0.5. At least 3 embryos or adult brains of each genotype were analyzed at each stage. Embryos and postnatal brains were fixed overnight in 4% paraformaldehyde, rinsed thoroughly in PBS and cryoprotected in 30% sucrose in PBS before sectioning at 12 μm on a cryostat. Adult brains were cryoprotected in 15% sucrose in PBS before sectioning at 35 μm and kept as free floating in PBS.

Immunohistochemistry

Primary antibodies were used at the following concentrations: rat anti-BrdU (1:250, Serotec), rabbit anti-Calbindin (1:2500, provided by P. Emson), goat anti-Calretinin (1:3000, Chemicon), rabbit anti-cleaved Caspase-3 (1:200, Cell Signaling), guinea pig anti-Doublecortin (Dcx) (1:3000, Chemicon), rabbit anti-Er81 (1:5000, provided by S. Morton and T. Jessell), rabbit anti-GAD67(1:2000, Chemicon), rabbit anti-GFP conjugated to Alexa Fluor 488 (1:500, Molecular Probes), guinea pig anti-GLAST (1:2000, Chemicon), rabbit anti-Nestin (1:200, provided by R. McKay), goat anti-Pax6 (1:200, Santa Cruz), rabbit anti-Pax6 (1:333, Covance), rabbit anti-phospho-Histone H3 (PPH3) (1:200, Upstate Biotechnology), rabbit anti-Tbr-1 (1:3000, provided by R. Hevner), guinea pig anti-Tbx21 (1:5000, provided by Y. Yoshihara), mouse anti-tyrosine hydroxylase (TH) (1:400, Chemicon). The Sp8 antibody was raised in rabbits against the C-terminal peptide, PEPGHRNGLE (Treichel et al., 2003). After affinity purification, this antibody was used at a 1:500 dilution. The secondary antibodies used for bright-field staining were: biotinylated swine anti-rabbit antibodies (1:200, DAKO), biotinylated horse anti-goat antibodies (1:200, Vector Laboratories). The ABC kit (Vector Laboratories) was used to visualize the reaction product using

diaminobenzidine (DAB, Sigma) as the final chromagen. In some cases nickel chloride was added to the DAB at 0.5% to enhance the signal. The secondary antibodies for fluorescent staining were; donkey anti-goat antibodies conjugated to Cy3 or Cy5 (Jackson Immunoresearch), donkey anti-mouse IgG antibodies conjugated to Cy2 (Jackson Immunoresearch), donkey anti-rabbit antibodies conjugated to Cy2,Cy3, or Cy5 (Jackson Immunoresearch), goat anti-Rat Alexa Fluor 594 (1:250, Molecular Probes). For the Sp8 and calbindin double staining, Sp8 cross-reaction between the two antibody complexes was blocked by an excess of unconjugated Fab fragments (Jackson Immunoresearch), as previously described (Toresson et al., 2000b). NeuroTrace green fluorescent Nissl stain (1:500, Molecular Probes) was used as a counterstain.

Bromodeoxyuridine Labeling

We injected postnatal day 4 mice intraperitoneally with bromodeoxyuridine (BrdU) (100 mg/kg, Sigma) and subsequently killed the animals 2 hr later. Embryos were processed as described above. Cyrosectioned tissue was treated with 4N HCL for 15 min at 65ºC, washed 3 times in PBS at room temperature, and incubated in 10% normal goat serum for 30 min. Immunohistochemistry was performed using Rat anti-BrdU (1:250, Serotec).

TUNEL Labeling

TUNEL labeling was performed as previously described (Stenman et al., 2003c). TUNEL positive cells were counted in the olfactory bulb and RMS at P12 in control and *Sp8* conditional mutant mice. Statistics were performed between the control and conditional mutants using a Student's unpaired t test.

In Situ Hybridization

In situ hybridization was performed as previously described (Toresson et al., 1999) using the *Sp8* probe (Bell et al., 2003).

Quantification

GAD67-positive cells in the inner layer (forming GCL) and outer layer (forming GL) were counted in every olfactory bulb section at E18.5 in control and *Sp8* conditional mutant embryos. Calretinin, TH and calbindin expressing cells were quantified at 400X magnification in the GL at P12 by counting three randomly chosen fields of each olfactory bulb section (5 to 9 sections were analyzed per animal) for control and *Sp8* conditional mutant mice. The average number of calretinin, TH or calbindin expressing cells was determined per field for each animal. Pax6 cells were counted in the SVZ or RMS of control and conditional mutants at 400X magnification. SVZ cells were counted at striatal levels. Cleaved Caspase-3 cells were counted in all LGE sections of controls and *Sp8* conditional mutants. For double staining quantification of Sp8 and interneuron markers, double and single labeled cells were counted in two separate areas of each section for a total of four olfactory bulb sections in at least two animals. Statistics were performed between the control and conditional mutants using a Student's unpaired t test.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/49/4/503/DC1/.

Acknowledgments

We thank A. Puche for sending the GAD_{65}-GFP mice and P. Emson, R. Hevner, R. McKay, T. Jessell, S. Morton and Y. Yoshihara for providing antibodies. We also wish to thank C. Schreiner for technical assistance and W.J. Scott for helpful discussions. This work was supported by NIH grants NS044080 and MH069643. Z.A. is supported by an NIH training grant (ES07051).

Received: June 28, 2005
Revised: December 7, 2005
Accepted: January 23, 2006
Published: February 15, 2006

References

Alvarez-Buylla, A., and Garcia-Verdugo, J.M. (2002). Neurogenesis in adult subventricular zone. J. Neurosci. *22*, 629–634.

Bell, S.M., Schreiner, C.M., Waclaw, R.R., Campbell, K., Potter, S.S., and Scott, W.J. (2003). Sp8 is crucial for limb outgrowth and neuropore closure. Proc. Natl. Acad. Sci. USA *100*, 12195–12200.

Bulfone, A., Wang, F., Hevner, R., Anderson, S., Cutforth, T., Chen, S., Meneses, J., Pedersen, R., Axel, R., and Rubenstein, J.L. (1998). An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons. Neuron *21*, 1273–1282.

Corbin, J.G., Gaiano, N., Machold, R.P., Langston, A., and Fishell, G. (2000). The Gsh2 homeodomain gene controls multiple aspects of telencephalic development. Development *127*, 5007–5020.

De Marchis, S., Temoney, S., Erdelyi, F., Bovetti, S., Bovolin, P., Szabo, G., and Puche, A.C. (2004). GABAergic phenotypic differentiation of a subpopulation of subventricular derived migrating progenitors. Eur. J. Neurosci. *20*, 1307–1317.

Dellovade, T.L., Pfaff, D.W., and Schwanzel-Fukuda, M. (1998). Olfactory bulb development is altered in small-eye (Sey) mice. J. Comp. Neurol. *402*, 402–418.

Doetsch, F., and Alvarez-Buylla, A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. Proc. Natl. Acad. Sci. USA *93*, 14895–14900.

Doetsch, F., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J. Neurosci. *17*, 5046–5061.

Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (2002). EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron *36*, 1021–1034.

Hack, M.A., Saghatelyan, A., de Chevigny, A., Pfeifer, A., Ashery-Padan, R., Lledo, P.M., and Gotz, M. (2005). Neuronal fate determinants of adult olfactory bulb neurogenesis. Nat. Neurosci. *8*, 865–872.

Hill, R.E., Favor, J., Hogan, B.L., Ton, C.C., Saunders, G.F., Hanson, I.M., Prosser, J., Jordan, T., Hastie, N.D., and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature *354*, 522–525.

Hinds, J.W. (1968). Autoradiographic study of histogenesis in the mouse olfactory bulb. I. Time of origin of neurons and neuroglia. J. Comp. Neurol. *134*, 287–304.

Kawakami, Y., Esteban, C.R., Matsui, T., Rodriguez-Leon, J., Kato, S., and Belmonte, J.C. (2004). Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. Development *131*, 4763–4774.

Kohwi, M., Osumi, N., Rubenstein, J.L., and Alvarez-Buylla, A. (2005). Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. J. Neurosci. *25*, 6997–7003.

Kosaka, T., Hataguchi, Y., Hama, K., Nagatsu, I., and Wu, J.Y. (1985). Coexistence of immunoreactivities for glutamate decarboxylase and tyrosine hydroxylase in some neurons in the periglomerular region of the rat main olfactory bulb: possible coexistence of gamma-aminobutyric acid (GABA) and dopamine. Brain Res. *343*, 166–171.

Kosaka, K., Aika, Y., Toida, K., Heizmann, C.W., Hunziker, W., Jacobowitz, D.M., Nagatsu, I., Streit, P., Visser, T.J., and Kosaka, T. (1995). Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb. Neurosci. Res. *23*, 73–88.

Kosaka, K., Toida, K., Aika, Y., and Kosaka, T. (1998). How simple is the organization of the olfactory glomerulus?: the heterogeneity of so-called periglomerular cells. Neurosci. Res. *30*, 101–110.

Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science *264*, 1145–1148.

Luskin, M.B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron *11*, 173–189.

Menezes, J.R., Smith, C.M., Nelson, K.C., and Luskin, M.B. (1995). The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. Mol. Cell. Neurosci. *6*, 496–508.

Parras, C.M., Galli, R., Britz, O., Soares, S., Galichet, C., Battiste, J., Johnson, J.E., Nakafuku, M., Vescovi, A., and Guillemot, F. (2004). Mash1 specifies neurons and oligodendrocytes in the postnatal brain. EMBO J. *23*, 4495–4505.

Penberthy, W.T., Zhao, C., Zhang, Y., Jessen, J.R., Yang, Z., Bricaud, O., Collazo, A., Meng, A., and Lin, S. (2004). Pur alpha and Sp8 as opposing regulators of neural gata2 expression. Dev. Biol. *275*, 225–234.

Pencea, V., and Luskin, M.B. (2003). Prenatal development of the rodent rostral migratory stream. J. Comp. Neurol. *463*, 402–418.

Puelles, L., Kuwana, E., Puelles, E., Bulfone, A., Shimamura, K., Keleher, J., Smiga, S., and Rubenstein, J.L. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J. Comp. Neurol. *424*, 409–438.

Stenman, J., Toresson, H., and Campbell, K. (2003a). Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. J. Neurosci. *23*, 167–174.

Stenman, J., Yu, R.T., Evans, R.M., and Campbell, K. (2003b). Tlx and Pax6 co-operate genetically to establish the pallio-subpallial boundary in the embryonic mouse telencephalon. Development *130*, 1113–1122.

Stenman, J.M., Wang, B., and Campbell, K. (2003c). Tlx controls proliferation and patterning of lateral telencephalic progenitor domains. J. Neurosci. *23*, 10568–10576.

Stoykova, A., Treichel, D., Hallonet, M., and Gruss, P. (2000). Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. J. Neurosci. *20*, 8042–8050.

Szucsik, J.C., Witte, D.P., Li, H., Pixley, S.K., Small, K.M., and Potter, S.S. (1997). Altered forebrain and hindbrain development in mice mutant for the Gsh-2 homeobox gene. Dev. Biol. *191*, 230–242.

Toresson, H., and Campbell, K. (2001). A role for Gsh1 in the developing striatum and olfactory bulb of Gsh2 mutant mice. Development *128*, 4769–4780.

Toresson, H., Mata de Urquiza, A., Fagerstrom, C., Perlmann, T., and Campbell, K. (1999). Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. Development *126*, 1317–1326.

Toresson, H., Potter, S.S., and Campbell, K. (2000a). Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development *127*, 4361–4371.

Toresson, H., Parmar, M., and Campbell, K. (2000b). Expression of Meis and Pbx genes and their protein products in the developing telencephalon: implications for regional differentiation. Mech. Dev. *94*, 183–187.

Treichel, D., Schock, F., Jackle, H., Gruss, P., and Mansouri, A. (2003). mBtd is required to maintain signaling during murine limb development. Genes Dev. *17*, 2630–2635.

Wichterle, H., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1997). Direct evidence for homotypic, glia-independent neuronal migration. Neuron *18*, 779–791.

Wichterle, H., Turnbull, D.H., Nery, S., Fishell, G., and Alvarez-Buylla, A. (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. Development *128*, 3759–3771.

Yoshihara, S., Omichi, K., Yanazawa, M., Kitamura, K., and Yoshihara, Y. (2005). Arx homeobox gene is essential for development of mouse olfactory system. Development *132*, 751–762.

Yun, K., Potter, S., and Rubenstein, J.L. (2001). Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. Development *128*, 193–205.

Zhao, C., and Meng, A. (2005). Sp1-like transcription factors are regulators of embryonic development in vertebrates. Dev. Growth Differ. *47*, 201–211.